Clinical, genetic and functional characteristics of three novel CYP17A1 mutations causing combined 17alpha-hydroxylase/17,20-lyase deficiency

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Clinical, Genetic and Functional Characteristics of Three Novel *CYP17A1* Mutations Causing Combined 17α-Hydroxylase/17,20-Lyase Deficiency

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Key Words
Steroidogenesis · P450c17 · Mutation analysis · Intersex · Adrenal gland

Abstract
Background: P450c17 has two distinct activities: 17α-hydroxylase activity and 17,20-lyase activity. Combined 17α-hydroxylase/17,20-lyase deficiency leads to a severe defect in the production of cortisol and sex steroids. In affected males this results in impaired masculinization with ambiguous or female external genitalia. Female patients have normal genitalia but show a lack of pubertal development in adolescence. An increased production of mineralocorticoids often leads to hypertension and hypokalemia in both sexes. ***Methods:*** To better understand the mechanisms of P450c17 deficiency, we studied 2 patients (both 46,XY) with combined 17α-hydroxylase/17,20-lyase deficiency of different severity: one with complete lack of masculinization and one with ambiguous genitalia. ***Results:*** Four mutations were identified by sequencing of the *CYP17A1* gene: I332T and A355T in the less severely affected patient; G111S and R440H in the patient with complete lack of masculinization. The three novel mutations were expressed in COS1 cells and all mutant proteins except I332T showed a complete loss of both enzymatic activities. I332T retained some residual 17α-hydroxylase as well as 17,20-lyase activity. ***Conclusion:*** We identified 2 patients with the phenotypical spectrum of P450c17 deficiency. Three novel mutations in the *CYP17A1* gene were identified and their functional characterization provided a good phenotype-genotype correlation. The location of the mutated residues in the three-dimensional model of P450c17 gave some additional insights into its structure-function relationship.

Introduction

The enzyme P450c17 plays an important role in steroid production. It is essential for the synthesis of cortisol as well as the production of sex steroids. P450c17 has two distinct activities: 17α-hydroxylase activity and 17,20-lyase activity, and is encoded by a single gene, *CYP17A1*, which consists of 8 exons [1; accession No. HUMP45C17S]. Mutations in the gene *CYP17A1* leading to P450c17 deficiency were first described 40 years ago by Biglieri et al. [2] in a female and New [3] in a male. Since then, mutations throughout the gene have been reported including rare cases of isolated 17,20-lyase deficiency [for details on allelic variants, see OMIM 609300]. However, in most cases of P450c17 deficiency, both activities are reduced. Such

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Table 1. Plasma basal and stimulated hormones

Hormone	Patient 1 (7 months)			Patient 2 (14 years)		
	basal	normal values	after hCG stimulation	basal	normal values[1]	after ACTH stimulation
LH, U/l	2.5	0.0–1.0	n.a.	48.6	5–25	n.a.
FSH, U/l	35	0.0–28	n.a.	93	4–20	n.a.
Progesterone, nmol/l	22.9	0.09–1.0	n.d.	32	0.3–1.5	65
17-OH-progesterone, nmol/l	5.1	0.3–9.5	5.3	0.74	0.3–4.7	0.76
DHEA, nmol/l	0.6	0.9–4.5	3.02	4.67	7.8–21.2	3.97
Androstenedione, nmol/l	0.33	0.5–2.5	0.8	0.1	1.4–7.9	0.1
Testosterone, nmol/l	0.1	4–14	1.1	<0.1	0.31–0.83	0.1
Estradiol, pmol/l	22	30–65	n.d.	<20	72–250	n.d.
Cortisol, nmol/l	82	70–550	n.a.	15	70–550	60
ACTH, ng/l	40	10–55	n.a.	257	10–55	n.a.
(Tetrahydro)corticosterone (THB), μg/24 h	*		n.a.	8,450	0–319	n.d.
Allo-THB, μg/24 h	*		n.a.	1,1470	84–1,034	n.d.
16-OH-pregnenolone	*		n.a.	1,991	0–35	n.d.

n.a. = Not applicable; n.d. = not determined; * = see qualitative profile.
[1] From Wudy and Homoki [14].

combined 17α-hydroxylase/17,20-lyase deficiency usually leads to a severe defect in the production of cortisol and sex steroids. In affected males this results in impaired masculinization with ambiguous or female external genitalia. Female patients have normal genitalia but show a lack of pubertal development in adolescence. An increased production of mineralocorticoids often leads to hypertension and hypokalemia in both sexes [for reviews, see 4, 5].

We present clinical and molecular data of 2 patients with combined 17α-hydroxylase/17,20-lyase deficiency of different severity. Three novel mutations were identified and their functional consequences were investigated in expression studies.

Materials and Methods

Patients

Patient 1 was a 7-week-old infant assigned to the female sex at birth who was referred for gender evaluation because of ambiguous genitalia with an enlarged clitoris and posterior labial fusion (Prader III) and palpable gonads in the labia majora. The karyotype was 46,XY. Hormones measured in plasma and urine in basal and stimulated (hCG) conditions suggested 17α-hydroxylase/17,20-lyase deficiency: low basal and stimulated androgens (DHEA, androstenedione and testosterone), high progesterone and high mineralocorticoid precursors (deoxycorticosterone DOC and corticosterone B, and progesterone metabolites 16-OH-pregnenolone).

Treatment was initiated with 3 cycles of 50 mg testosterone depot, whereafter penile size increased from 5 to 37 mm allowing the surgeon to correct the genital defect and the patient was reassigned to the male sex at 4 months of age.

Patient 2 was raised as a girl and accidentally diagnosed at age 14 years when, during an appendectomy, no uterus and tubes were seen, hypertension with a blood pressure around 140/90 mm Hg and low potassium (3.0 mmol/l) were noted. She had no breast development and normal prepubertal female external genitalia but a blind-ending vagina. Due to bone age retardation, adult height prediction was increased to 187 cm. The karyotype was 46,XY. Hormone values including an ACTH test were compatible with complete combined 17α-hydroxylase/17,20-lyase deficiency. Undescended testes were found and subsequently removed.

The patient was treated with hydrocortisone, which had some lowering effect on blood pressure but nevertheless did not lead to satisfactory control of hypertension. Therefore, additional treatment with spironolactone was necessary. The patient suffered from a depressive mental disorder and headaches but neither replacement therapy with hydrocortisone nor addition of estradiol to initiate pubertal development improved well-being. As the patient increased by herself hydrocortisone until overdose, the treatment was stopped. Upon suspension of hydrocortisone a rise of corticosterone and a very high deoxycorticosterone level were noted, but adequacy of adrenal therapy with spironolactone alone was documented by normal blood pressure and normal plasma concentrations of potassium and ACTH. Yet after discontinuation of hydrocortisone, the patient felt even more depressed and demanded to return to hydrocortisone treatment in addition to the above-mentioned therapy. The hormone values of both patients are summarized in table 1 and figure 1a.

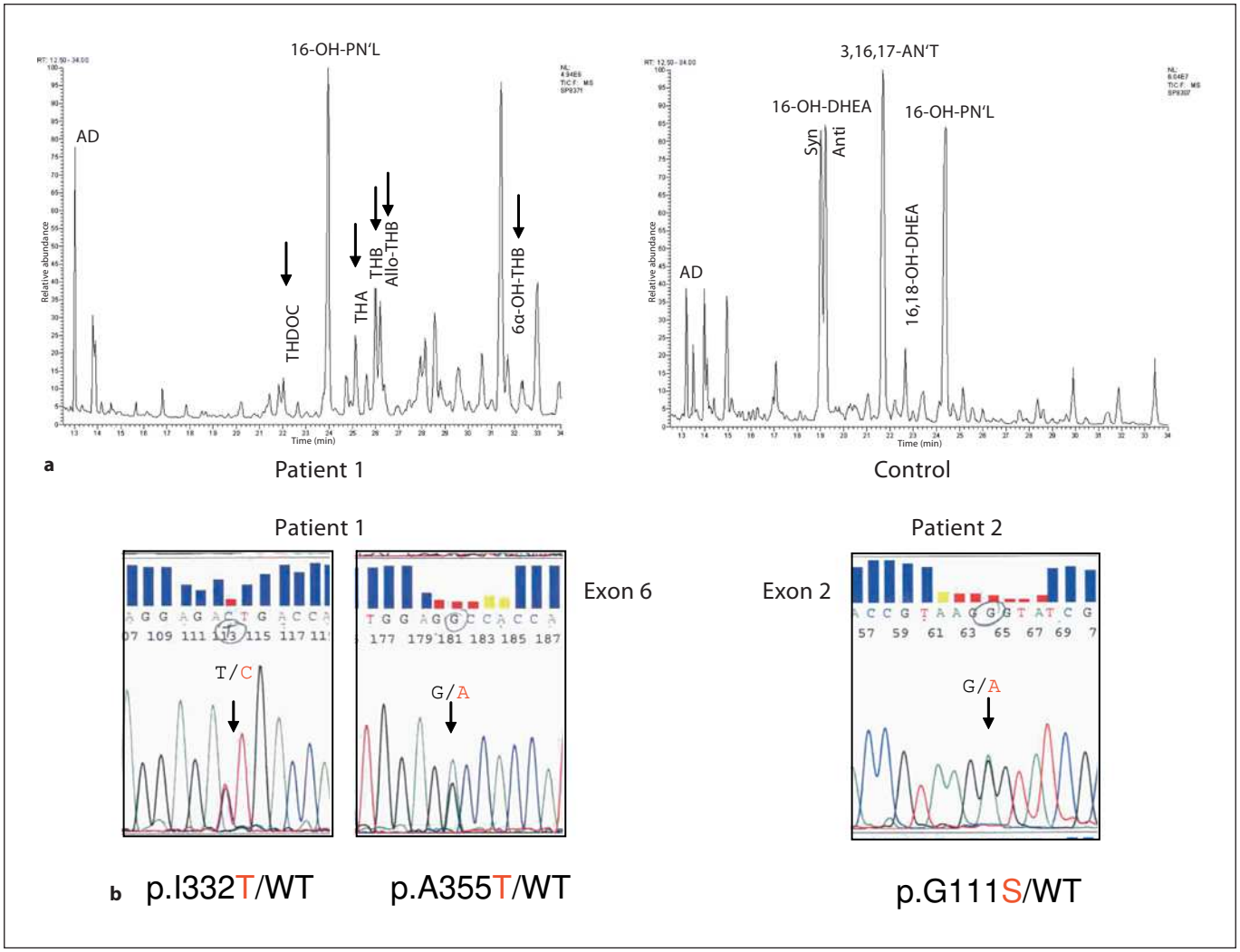


Fig. 1. a Qualitative urinary steroid profile of patient 1 compared to an age-matched control (Control). Only the peaks essential for the diagnosis (arrows) are labeled. The mineralocorticoid precursors tetrahydrodeoxycorticosterone (THDOC) tetrahydrocompound A (THA), tetrahydrocorticosterone (THB) and allo-THB are significantly high (and absent in the normal control). Compatible with an androgen synthesis defect, all the androgens in particular DHEA metabolites (16-OH-DHEA and isomers) are absent in patient 1 and very high in the normal control infant. IS = Internal standard (androstanediol). **b** DNA sequence chromatograms obtained by direct sequencing of *CYP17A1* PCR products showing the presence of the heterozygote c.T1055C and c.G1123A both in exon 6 leading to p.I332T and p.A355T, respectively, in patient 1, and c.G391A in exon 2 of patient 2 leading to p.G111S. The c.G1379A (p.R440H) mutation was not further characterized and is not shown.

Mutation Analysis

Genomic DNA was extracted from peripheral blood leukocytes using the Qiagen DNA blood and cell culture kit (Qiagen GmbH, Hilden, Germany) and used to perform polymerase chain reaction (PCR) exonic amplification of the *CYP17* gene as previously described [6]. The PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Kit and analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems; reference sequence: HUMP45C17S [cf. 1]). Primer sequences are available upon request.

Expression Studies

Wild-type *CYP17A1* cDNA (originally from Michael R. Waterman), after addition of an N-terminal myc-tag, was inserted into a pcDNA3.1 vector. Mutant cDNAs were constructed using the QuikChange II site-directed mutagenesis kit from Stratagene. Introduction of the mutations was confirmed by sequencing. Wild-type or mutant cDNA was transiently transfected into confluent COS-1 cells using TransFast transfection reagent (32 μl transfection reagent/3.6 μg DNA). To standardize the steroid production, cells were cotransfected 1:2 with BGal. Forty-eight hours after transfection, steroidogenic precursors (progesterone for 17α-hy-

droxylase activity and 17-OH-pregnenolone for 17,20-lyase activity) were added at concentrations of 0.1 and 1.0 μM. Six hours after addition of the precursor, supernatants were removed and kept frozen at –20°C until measured. β-Galactosidase activity was measured using the β-galactosidase enzyme assay system (Promega). The secreted steroids, 17-OH-progesterone (reflecting 17α-hydroxylase activity) and DHEA (reflecting 17,20-lyase activity), were measured in duplicates by radioimmunoassay using commercial kits from Diagnostic System Laboratories (Morwell Diagnostics GmbH, Egg, Switzerland). Values were standardized for β-galactosidase activity and expressed as percentage of wild-type activity.

All values are expressed as mean ± SD and represent the results of three independent experiments.

For mutation, I332T and the wild-type K_m and V_{max} were assayed by exposing the transfected cells to six different precursor concentrations ranging from 0.1 to 30 μM. The products were measured as described above. K_m and V_{max} were calculated using GraphPad Prism 4. Immunofluorescence was performed as previously described [7].

Results

In patient 1, sequencing of the *CYP17A1* gene revealed the presence of a compound heterozygosity consisting of a c.T1055C and c.G1123A (reference mRNA NM_000102) leading to p.I332T and p.A355T respectively (fig. 1b). The parents are heterozygote (not shown). In order to assess the functional consequence of the mutations, COS1 cells were transfected with the expression vector pcDNA3 containing either wild-type or mutant *CYP17A1* cDNA.

The A355T mutant protein expressed in COS1 cells showed a complete loss of 17α-hydroxylase as well as 17,20-lyase activity. In contrast, compared to the wild-type protein, the I332T mutant seems to retain 15–25% of 17α-hydroxylase and about 10% of 17,20-lyase activity at two different substrate concentrations (0.1 and 1.0 μmol/l; see fig. 3). The residual activity of the I332T mutant was therefore further explored by using six different precursor concentrations in the transfected COS1 cells, and although classical enzyme kinetic is not possible in a whole-cell system, apparent K_m and V_{max} were calculated from the resulting concentrations of 17-OHP and DHEA. The results confirm that I332T retains some 17α-hydroxylase (K_{cat} WT vs. Mut: 79 vs. 4, vector 0.5) as well as some 17,20-lyase activity (K_{cat} WT vs. Mut: 128 vs. 10, vector incalculable), where the relative residual activity of the mutant is lower with higher precursor concentrations (fig. 2; table 2).

Patient 2 was also found to be a compound heterozygote carrying a c.G391A in exon 2 with the consequent replacement of glycine-111 by serine (p.G111S; fig. 1b) as well as a c.G1379A substitution resulting in the R440H mutation. R440H has been characterized already in a patient with severe P450c17 deficiency and has been shown to have no 17α-hydroxylase or 17,20-lyase activity [8]. Also the G111S mutation when expressed in COS1 cells lead to the synthesis of a protein with no residual activity.

Immunofluorescence microscopy demonstrated that the mutant proteins (G111S, A355T and I332T) were expressed in transfected COS1 cells in a fashion comparable to the wild-type protein, suggesting that the loss of enzymatic activity is not due to defects of synthesis, stability or localization of the P450c17 proteins. COS1 cells transfected with the empty pcDNA3.1 vector did not express the P450c17 protein (fig. 3).

Discussion

Patient 1 is a compound heterozygote bearing the mutations A355T and I332T. A355T showed no activity in our expression studies in COS1 cells. However, mutation I332T seems to retain some residual activity (about 15% of 17α-hydroxylase and 10% of 17,20-lyase activity). In analogy with what was observed for CAH due to 21-hydroxylase deficiency, where compound heterozygotes for two different *CYP21* mutations usually have a phenotype compatible with the presence of the milder of the gene defects [9], it is feasible to hypothesize that the milder phenotype in this patient (subtle signs of masculinization) is due to the presence of the I332T milder mutation. Residual activity in this mutation is not totally surprising as, according to the published structure of P450c17 [10, 11], I332 is not located within the active site. However, being near the end of the J helix it is not far upstream of the redox partner binding site and might well lead to some distortion of the redox partner binding surface resulting in the observed partial loss of activity. Similar to the E331del mutation we previously described [7] and which also showed some residual activity, the effect of I332T on downstream structures might be dampened by the relatively unstructured region at 337–341 between the J helix and the J′ helix. A355T mutation is located in the K helix which is part of the redox partner binding site. It might have been expected to lead to a preferential loss of lyase activity with some hydroxylase activity preserved in congruence to the reported mutations at R347 and R358, which were associated with isolated lyase deficiency [12]. However, according to our expression studies, A355T seems to disrupt the structure of the protein so severely that all enzymatic activity is lost.

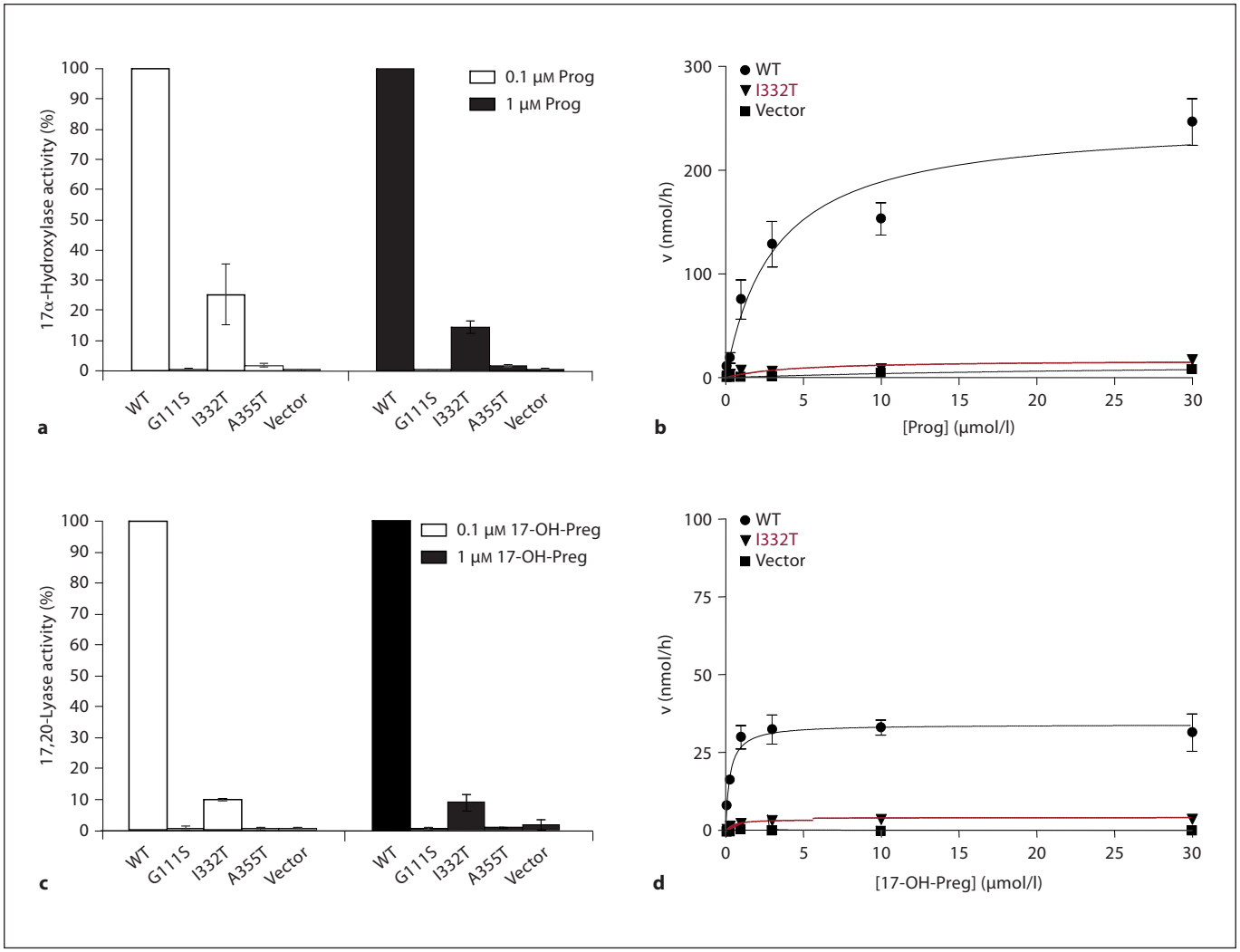
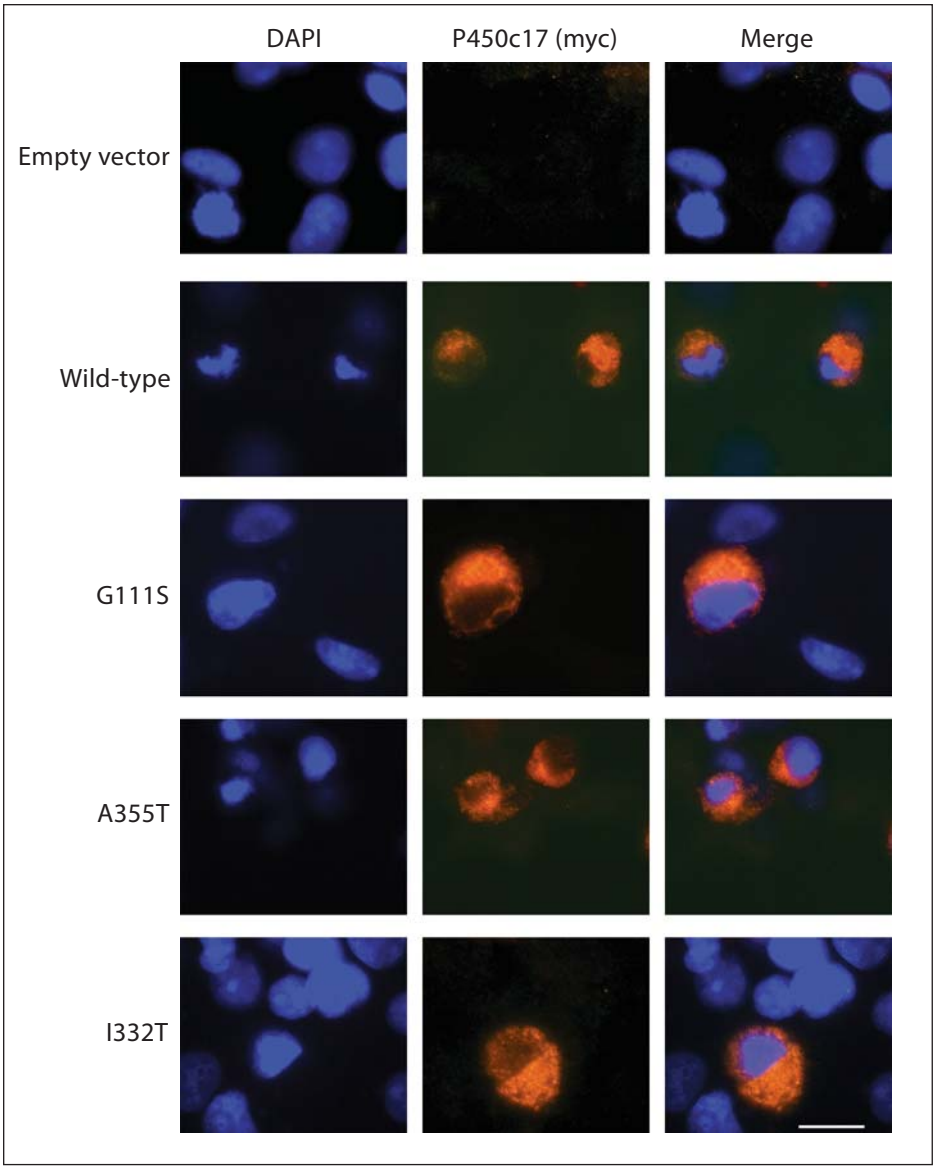


Fig. 2. Production of 17-OH-progesterone as a measure of 17α-hydroxylase activity (**a**) and DHEA (**c**) as a measure of 17,20-lyase activity after 6 h incubation with different concentrations (0.1 and 1 μM) of precursors (progesterone and 17-OH-pregnenolone, respectively) in COS1 cells transfected with empty vector (vector), wild-type (WT) and mutated *CYP17A1* cDNAs. The data represent mean ± SD of three independent experiments. For WT, vector and I332T mutant, apparent K_m and V_{max} were calculated from the resulting concentrations of 17-OHP (**b**) and DHEA (**d**) after exposing transfected COS1 cells to different concentrations of precursors (0.1, 0.3, 1, 3, 10 and 30 μM).

Table 2. Kinetic of p.I332T

	17α-Hydroxylase			17,20-Lyase		
	WT	I332T	vector	WT	I332T	vector
V_{max}, min^{-1}	248	17.1	15.5	34.6	4.1	0.043
K_m, μmol	3.15	4.24	30	0.27	0.39	incalculable
K_{cat}	78.7	4.03	0.5	128	10.5	incalculable



Color version available online

Fig. 3. Localization of wild-type and mutant myc-tagged *CYP17A1* cDNAs in transfected COS1 cells using an anti-myc antibody. Cells transfected with an empty vector serve as negative control. Scale bar: 20 μm.

In patient 1 an additional difficulty was the decision about gender reassignment and whether micropenis therapy could be successful. Rapid diagnosis and involvement of a multidisciplinary team allowed us finally to reassign the patient to the male sex at the age of 4 months. Treatment with 3 cycles of 50 mg testosterone depot resulting in an increase of penile size from 5 to 37 mm allowed the surgeon to correct the genital defect.

Our patient 2 clinically and biochemically corresponds to complete 17α-hydroxylase/17,20-lyase deficiency. The severity of the phenotype is not surprising in light of the mutations found in this patient: G111S and R440H. The mutation R440H has been reported earlier in a patient of German origin with complete 17α-hydroxylase deficiency and has been shown to lead to the synthesis of a protein with no detectable 17α-hydroxylase or 17,20-lyase activity [8]. In our expression studies the mutation G111S also caused a complete loss of both 17α-hydroxylase and 17/20-lyase activities. G111S is located in the B′-C loop, a region of the protein that seems to be equally sensitive being part of the substrate entrance with the neighboring highly conserved I112 which is important for protein function [11, 13]. In fact, displacement of I112 has been shown to completely destroy protein function [10]. Sub-

stitution of glycine-111 by serine might displace I112 enough to abolish all enzymatic activity probably by disrupting substrate binding. The importance of residue 111 is confirmed by the severe phenotype and the report that a similar constructed mutant, G111D, completely lost enzymatic activity [13].

Although she presented as a typical case of 17α-hydroxylase/17,20-lyase deficiency, management of patient 2 proved to be difficult as, due to the rarity of the disorder, no real consensus on therapy exists. These patients do not suffer from cortisol deficiency and therefore do not need any cortisol substitution as the elevated corticosterone is sufficient to act as a substitute for cortisol. However, in order to treat the hypertension, low doses of glucocorticoids may be given in young children as their DOC levels should be readily suppressible with less than physiologic replacement doses. If a patient has not received glucocorticoids in childhood and has had long-term DOC excess, the hypertension may become 'fixed' and be no longer sensitive to low-dose glucocorticoids and hence requires a mineralocorticoid antagonist such as spironolactone or the promising eplerenone which effectively prevent the blood pressure-increasing effect of the abundant mineralocorticoid precursors.

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